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Applicant:	McDonald *et al.*.	Group Art Unit:	1646
Serial No.:	09/360,242	Examiner:	Landsman, R.
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For: *METHODS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND DISORDERS*

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, JOHN R. McDONALD, declare as follows:

1. I am an inventor of and am familiar with the subject matter of the above-captioned application; and I have read the Office Action, mailed March 2, 2000, in connection with the above-captioned application.

2. I received B.Sc. and Ph.D. degrees at Napier College, Edinburgh, completed successful postdoctoral appointments in Canada and The United States before leaving academia for the biotechnology industry (Boulder CO, and San Diego CA). I have been involved in all aspects of the Research and Development process from project planning through IND filing. My research has focused upon growth factor signal transduction, multiple sclerosis, and the purification and characterization of neurotrophic factors and growth factor-mitotoxin fusion proteins. I have received several peer-reviewed awards and grants, including a US National Institutes of Health Small Business Innovation Research Grant. I am co-author of over fifty publications, and a named inventor on seven patent applications.

3. I am a founder of Osprey Pharmaceuticals Limited, Canada, and I am currently Vice-President Research & Development and a Director at the company. The conjugates described and claimed in the above-captioned application are

broad-based, widely applicable anti-immunoinflammatory drugs for treatments, including treatment secondary tissue damage-associated disorders including those that accompany central nervous system trauma and disease, including spinal cord injury, head injury, multiple sclerosis, amongst others, and for other inflammation-driven diseases as divergent as asthma, arthritis, HIV and cancer.

4. In my capacity as a Director, I have directed the experiments described below, which demonstrate the effectiveness of conjugates of chemokine receptor-targeting agents for treatment of diseases that are characterized or caused by a pathophysiological inflammatory response. These conjugates provide a more selective and targeted delivery than previous ligand-directed delivery conjugates. The following exemplary results evidence these properties. The following discussion contrasts the results with prior ligand toxins and also points out the advantages, which are also described in the above-captioned application, of the conjugates and methods of the above-captioned application.

The data indicate that the chemokine-toxin conjugates target cells with specificity and a certain degree of predictability. In addition, the data indicates that these agents distinguish between activated and quiescent cells and that potential toxic side effects may be a non-issue or at most, minimal.

With these considerations in mind and the current knowledge regarding the complex nature of the temporal and spatial participation of chemokines, chemokine receptors and leukocyte subtypes in disease and trauma, specific chemokine receptor targeting conjugates should be effective in treating a wide array of conditions (see, *e.g.*, Table 2 in the specification of the above captioned application).

Background, experiments, results, discussion and conclusions

The conjugates provided in the above-captioned application permit selective, deliberate, and surreptitious delivery of a therapeutic agent, such as a cytotoxin to cells that orchestrate and perpetuate immune-mediated secondary tissue damage and to cells that are specifically activated in other disease states.

The conjugates are targeted by agents, such as chemokines, that specifically bind to chemokine receptors, which are expressed on cells involved in a variety of disease states, particularly states involving pathophysiological inflammatory response that cause secondary tissue damage. There is a close causal relationship between the type, number and activity of these cells, the amount of secondary damage, and the severity of a given trauma or disease state.

By attaching a cytotoxic or cell inhibitory moiety to an appropriate chemokine or other agent that specifically binds to chemokine receptors and internalizes linked targeted agent, the therapeutic conjugate can be delivered to the heart of the trauma or disease, such as the proliferating microglia in the injured central nervous system (CNS), eosinophils in the lung of the ARDS patient, or monocytes, neutrophils and T-cells in the arthritic joint. Furthermore, using a leukocyte-directed chemokine receptor targeting conjugate to deliver a toxic agent overcomes many of the specificity problems that hindered the development of effective therapies using earlier developed ligand toxin fusion proteins, such as those in Volk *et al.* (1994) *J. Immunol.* 2497-2505 and Ogata *et al.* (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*:4215-4219, cited in the Office Action, mailed March 2, 2000.

Problems With prior Ligand-Toxin Therapy That Can Be Avoided By Therapy with the chemokine-receptor targeting conjugates

Prior approaches

The classical approach to drug development values specificity above all other attributes of the candidate compound. Typically, specificity has meant things like high affinity binding to a single receptor type or inhibition of a biochemical pathway at a specific point. Single pathway anti-inflammatory agents, no matter how specific, are at best partial solutions for all inflammatory conditions, except the mildest forms of inflammation. Another interpretation of

specificity holds that certain cell types (e.g. leukocytes or cancer cell clones) be selectively removed by the therapeutic agent. Unfortunately, presently available agents tested (e.g. monoclonal antibodies) are rarely able to distinguish the target cell from normal cells. A drug may have one hundred percent specificity for its binding site but little selectivity for the disease.

When a ligand-toxin lacks specificity for the target cells it is often administered more frequently and in higher doses to produce the desired therapeutic effect. In addition to the unwanted effect on bystander cells the chimeric protein can trigger an immune response which can lower the efficacy of subsequent treatments (see, *e.g.*, Uckun *et al.* (1997) *Clin Cancer Res* **3**, 325-37.). To prevent immunogenicity, some ligand-toxins have been administered in combination with immunosuppressants. For example, Rituxan, which depletes antibody producing B-cells, and is non-immunogenic itself, is currently being tested in combination with anti-LMB-1 immunotoxin for the treatment of solid tumor breast cancers.

Sometimes the problem with the ligand toxin is not a lack of selectivity for its receptor but a lack of selectivity of the target cell type for the disease. Thus the T-cell selective OX-40-ricin A immunotoxin has been mentioned as a possible treatment for autoimmune diseases such as MS, arthritis and GVHD (Weinberg *et al.* (1999) *J Immunol* **162**, 1818-26; and Weinberg, A.D. (1998) *Mol Med Today* **4**, 76-83). Since other leukocyte cell groups, including MNPs, are implicated in the pathology of MS it is unlikely to be effective. More importantly, a recent study has shown that OX-40 expressing T cells are not increased in clinically active MS (Hintzen *et al.* (2000) *Acta Neurol Scand* **101**, 57-60). The Diphtheria toxin containing chimeras, DAB486IL-2 and DAB389IL-2, which target activated T-cells have been tested in clinical trials for arthritis and psoriasis, respectively. The anti-arthritic effect was marginal (Moreland *et al.* (1995) *Arthritis Rheum* **38**, 1177-86; and Schrohenloher *et al.* (1996) *J Rheumatol* **23**, 1845-8), and there was no effect in psoriasis (DiSepio (1999) *Drug Design Today* **5**, 222-31).

Thus, the prior art ligand-toxins often suffer from a lack of specificity that leads to toxic and immunogenic side effects (Kreitman, R.J. (1999)*Curr Opin Immunol* **11**, 570-8; Frankel *et al.* (2000) *Clin Cancer Res* **6**, 326-334). A wide distribution of the target binding site on normal and diseased cells is the principal source of unwanted effects and systemic toxicity. This is certainly the case for most immunotoxins as none of their receptor targets are truly tumor specific for the diseased cells (Frankel *et al.* (2000) *Clin Cancer Res* **6**, 326-334). Non-specific or low affinity binding to a closely related receptor can also contribute to side effects.

Chemokine receptor targeting conjugates

Chemokine-receptor targeted conjugates avoid many of these problems. The chemokine receptor targeting conjugates (hereinafter referred to as the conjugates) provided in the above-captioned application exhibit exquisite selectivity and flexibility of design. No existing ligand-toxin fusion proteins have the same potential for therapy as the conjugates of the instant application.

The large number of ligands and receptors for the conjugates provided in the above-captioned application make it possible to choose a suitable combination of targeting agent and receptors of a desired distribution and expose target cells with a high degree of selectivity. Disease-related chemokine receptor upregulation increases the likelihood of successful chemokine receptor-targeting conjugate target cell interactions, which, as borne out by the experiments herein, should occur at relatively low concentrations of the drug. Furthermore, the exact chemokine receptor targeting agent can be selected to suit the stage and severity of the disease.

The immunogenic side effects observed with prior art ligand-toxins should not occur with these conjugates, since the conjugates target cells, such as activated leukocytes, that are present or are increased only in the disease state. Hence the conjugates provided herein may not exhibit immunogenic side-effects of other ligand conjugates. Data shown below supports this contention.

Materials and Methods

Materials and methods used in the experiments described herein are also set forth in the application.

Construction of Genes

Conjugates were constructed as described in the above-captioned application. As described above, conjugates with a variety of specificities were constructed. Exemplary of these constructs are OPL9810 (an MCP-1-Shiga toxin conjugate) and OPL9811 (an SDF-1β-shiga toxin conjugate.

Tissue culture protocols

Primary cultures

Protocols for adult human brain cell culture are performed as described in detail by Yong *et al.* ((1997) Culture of glial cells from human brain biopsies. In *Protocols for Neural cell Culture* (A. Richardson and S. Fedoroff, eds), Humana Press, St. Louis 157-172). Briefly, surgically resected brain tissue is cut into 1 mm cubes and incubated in 0.25% trypsin for one-hour at 37 °C. The suspension is passed through a 130 um nylon filter which dissociates the tissue into single cells. Following centrifugation (15,000 rpm, 25 min.) in 30% Percoll, the supernatant contains viable neurons while the pellet is comprised of tissue debris, myelin, and red blood cells. The neural cells are collected and plated onto uncoated tissue culture plastic. The cultures are incubated for 24 hours at 37^{o}C by which time the microglia adhere to the plastic while the oligodendrocytes remain in solution. Oligodendrocytes are decanted, centrifuged, and plated onto poly-L-lysine, to which they adhere. Neurons and astrocytes do not survive this isolation process, however, the resulting populations of oligodendroglia and microglia are greater than 95% pure.

Neurons and astrocytes are derived from fetal brain specimens. Brain tissue is cut into small cubes and incubated with 0.25% trypsin and 100 μg/mg DNAase at 37°C., as described in Oh *et al.* ((1996) Astrocytes promote process outgrowth by adult human oligodendrocytes in vitro through interaction between bFGF and astrocyte extracellular matrix. *Glia* 17, 237-53). The suspension is passed through a 130 um nylon filter and the filtrate is collected, washed, and seeded onto poly-L-lysine-coated tissue culture plastic to allow the cells to adhere. A Percoll centrifugation step is not required since most fetal axonal tracts are not myelinated. To purify the neuronal population the mixed culture is treated with 25 uM cytosine arabinoside (Sigma, St. Louis) which destroys the mitotically active astrocytes. To purify the astrocytic population the mixed culture is passaged in the presence of 0.25% trypsin, which kills neurons. Adult astrocytes are isolated in a similar manner.

In general, neural cell cultures are fed twice weekly with minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 20 μg/ml gentamicin, and 0.1% dextrose (Gibco, Grand Island, N. Y.).

Human peripheral blood leukocytes are harvested according to published methods (Chabot *et al.* (1997) Microglial production of TNF-alpha is induced by activated T lymphocytes. Involvement of VLA-4 and inhibition by interferonbeta-1b. *J Clin Invest* 100, 604-12.) In brief, venous blood is layered on to Ficoll-Hypaque (Pharmacia) and centrifuged for 30 min at 2500 rpm. The mononuclear cell fraction is collected, washed twice, and seeded onto uncoated tissue culture substrates. Two hours later, floating cells (mostly T lymphocytes) are removed to leave behind an adherent population that consists primarily of monocytes. These cells are used immediately in cytotoxicity experiments, or they are activated prior to experimentation (three days, 1 mg/ml anti-CD3 receptor ligation for T-cells or 1 mg/ml lipopolysaccharide for monocytes).

In general, all hemapoietic cells (primary cells, or the cell lines described below) are maintained in RPMI medium supplemented with 10% fetal bovine serum, 20 mg/ml Gentamicin and 0.1% dextrose (Gibco).

Cell lines

Cell lines derived from human mononuclear phagocytes are cultured using routine procedures. For example, monocyte-derived U937 and THP-1 cells, and the microglia-like CHME line from fetal brain (obtained from Dr. Tardieu, France). Human microglial cell lines established after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neurosci Lett* 195, 105-8), have been used to test conjugates prepared as described in the above-captioned application. Numerous cell lines, including those of astrocytic and neuronal lineage, can be readily obtained from the ATCC (Rockville, MD) and successfully cultured using the instructions that accompany the shipment.

Immunohistochemistry

Indirect immunohistochemistry is routinely performed to confirm the purity of enriched cultures, and by extension, to distinguish between different cell types in a mixed culture. There are a variety of academic and commercially available cell type-specific antibodies that can be used to facilitate this process. Examples include, an anti-galactocerebroside (GalC) antibody to identify oligodendrocytes, an anti-glial fibrillary acidic protein (GFAP) antibody for astrocytes, an anti-Mac-1 antibody for microglia, and an anti-neurofilament antibody for neurons (anti-NFL).

In brief, live cells on cover slips are treated with an appropriate fixative (e.g., 4% paraformaldehyde for galactocerebroside, and 95% ethanol/5% glacial acetic acid, v/v). A predetermined concentration of the primary antibody is applied followed by an appropriate secondary antibody (typically, rhodamine or fluorescein-conjugated goat anti-rabbit or anti-mouse IgG). The stained cells are examined using a microscope equipped to detect immunofluorescence. Analysis of adherent cell cultures primarily relies upon indirect immunohistochemical staining and labeling, and double labeling methods. Each cell type is counted in a sufficiently large number of randomly chosen microscope fields and the data are subjected to appropriate statistical analysis. Depending upon the mode and/or level of toxicity, that is to say, apoptosis versus necrosis and/or subtle versus gross toxicity, the degree of cell death is recorded either qualitatively (toxicity

grade of 0 to 4 (see, see example in Noble *et al.* (1994) Astrocytes and catalase prevent the toxicity of catecholamines to oligodendrocytes. *Brain Res* 633, 83-90) or quantitatively (the number of dead cells as a percentage of the total population (see example in, Oh *et al.* (1997) The promoting effects of bFGF and astrocyte extracellular matrix on process outgrowth by adult human oligodendrocytes are mediated by protein kinase C. *Brain Res* 757, 236-44). In most instances data are analyzed using a one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons. Suspended cells are analyzed using a flow cytometer (see, *e.g.*, Williams *et al.* (1997) *Brain Res* 754, 171-80; Stuve *et al.* (1996) *Ann Neurol* 40, 853-63; Stuve *et al.* (1997) *J Neuroimmunol* 80, 38-46; Williams *et al.* (1992) *J Neuropathol Exp Neurol* 51, 538-49), which automates both data collection and appropriate statistical analysis (e.g.equipment from Becton Dickinson).

Cytotoxicity Assays

Briefly, test cells are supplied with fresh medium containing control and test substances (at different concentrations) and incubated for a specified period (24-36h). Cytotoxicity is then measured as the ability of adherent cells to reduce the vital dye MTT (see, *e.g.*, Mosmann (1983) *J Immunol Methods* 65, 55-63 and Gieni *et al.* (1995) *J Immunol Methods* 187, 85-93). Cytotoxicity in suspended cell cultures is measured using a Coulter counter, where the absolute number of cells is taken as an index of the number of surviving cells per test condition. Finally, general cell survival and morphology are monitored throughout the experiments using phase inverted microscopy and exclusion of the dye trypan blue (Yong *et al.* (1997) Culture of glial cells from human brain biopsies. In *Protocols for Neural cell Culture* (A. Richardson and S. Fedoroff, eds), Humana Press, St. Louis 157-172).

Chemotactic Assays

The chemotactic effect of each conjugate is measured as a test of the biological activity of the ligand component. Several chemotactic assays are described in the literature and employed (see, *e.g.*,Stuve *et al.* (1996) *Ann Neurol* 40, 853-63; Stuve *et al.* (1997) *J Neuroimmunol* 80, 38-46). In brief, the top and bottom compartments of a modified Boyden chamber are separated by a 3 μm membrane coated with fibronectin. Hematopoietic responder cells, appropriate to the chemokine being tested, are placed into the top compartment of the chamber while test materials are placed in the bottom. After an appropriate period of time, the number of cells that have migrated in response to a chemotactic stimulus is recorded. Migrating T-lymphocytes fall off the membrane into the lower chamber and the can be counted using a Coulter counter. In contrast migrating MNPs are retained on the underside of the membrane, and consequently, the upper surface must be washed and the lower surface fixed, prior to staining with Coomassie Blue and analysis by light microscopy.

RESULTS AND DISCUSSION

Activated Leukocytes And Upregulated Chemokine Receptors Facilitate Therapy and permit low dosage administration

Under normal circumstances, the only practical way to influence the equilibrium between a ligand and a fixed population of target receptors is to increase the concentration of the ligand. In inflammatory conditions, however, the chemokine receptor population is increased. Consequently, the conjugates provided in the above-captioned application can be used at lower concentrations than previously prepared conjugates that target cells with only small increases in a disease state.

As demonstrated in experiments described below, the conjugates provided in this application target activated cells and not quiescent cells. As the results show, such conjugates are not likely to cause immunosuppression and patients are unlikely to develop drug resistance.

The following experiments demonstrate that receptor targeting conjugates distinguish between activated and quiescent target cells. It is the former that are associated with inflammatory damage. At concentrations of OPL98110 (around 1 μg/ml) that have little if any detectable effect on near confluent cultures of THP-1 cells, a cytotoxic effect that is selective for activated cells was demonstrated.

***In vitro* activity of OPL98110.**

OPL98110, which is a conjugate containing MCP-1 and shiga toxin, was prepared as described in the application. MCP-1 specifically binds to CCR2 receptors, which are present in the activated microglial cells in the CNS. Given its profile of cell and receptor selectivity OPL98110 (MCP-1, CCR2) is an appropriate chemokine-toxin conjugate for use in the nervous system. Our results demonstrated that it targets cells of monocytic lineage including THP-1 leukemia cells, primary human peripheral blood mononuclear cells (PBMCs) and T-cells. There was no evidence of an effect on primary human neurons or U251 cells (a glioma of astrocytic lineage). This chimera is effective on target cells within 24 hours at doses as low as 1 μg/ml and will kill up to 70% of the culture at doses between 5 and 10 μg/ml. Entire cultures are eradicated within 48 hours.

It appears that OPL98110 kills activated monocytoid cells in the short term ignoring the quiescent cells, which do not express the targeted receptor. not expressing receptor). As the quiescent cells become activated in culture (perhaps in response to released mediators from dead or dying cells) during the incubation period, the appropriate receptors are expressed and they too become targets.

OPL98110 Activity On Stationary Target Cells

Human peripheral blood monocytes (from healthy donors) and THP-1 cells (a human monocytic cell line) were treated with 1:10 and 1:50 dilutions of Control B and OPL98101. Twenty-four hours later the cells were examined by phase contrast microscopy and representative fields were photographed and counted. OPL98110 caused marked membrane disruption and vacuolization in both cell types. Most of the treated cells appeared abnormal, and an increased

amount of cellular debris indicated that some were already dead. At the lower concentration of the conjugate (1:50) 20-25% of both cell types were affected.

Note: Control A is tissue culture medium. Control B is a wash fraction obtained prior to the elution of the chemokine-toxin from the nickel-affinity resin. This fraction was heavily enriched in *E. coli* proteins. Unless otherwise indicated all procedures were carried out in triplicate.

Other experiments demonstrated that the conjugate targets cells of monocytic lineage (i.e. THP-1 cells which are microglia and MNP-like) as well as human peripheral blood monocytes and T-cells, but not primary human neurons or U251 cells (a glioma of astrocytic lineage). OPL98110 is effective within twenty-four hours at doses as low as 1 μg/ml and will kill the entire culture at doses between 5 and 10 μg/ml. A dose dependent effect of OPL98110 on activated cells (THP-1 and T-cells) is detectable below 1 μg/ml.

OPL98110 Activity On Proliferating Target Cells

In another experiment THP-cells were grown for 48 in the presence and absence of OPL98110 (1:10 dilution) and cell viability examined by either microscopy or the ability to exclude trypan blue. Cells that exclude the stain are alive while stained cells are dead. Since THP-1 cells are naturally non adherent, and in order to produce a more accurate count, control and treated cells were dissociated from cellular debris by gentle pipetting prior to counting. After 48 hours, 7.4 ± 3% of the control cells were dead (i.e. stained) in comparison to 58.8 ± 13% of the OPL98110 treated group. This is a 51.4% difference. Sister wells examined after 96 hours revealed that control cells had proliferated and continued to appear quite normal and healthy while the chemokine-toxin treated cultures contained a lot of cellular debris, but few if any live cells.

These cultures were split and allowed to incubate for a further seven days. Control THP-1 cells continued to thrive and proliferate. There were no surviving cells in wells split from OPL98110 treated cultures). These studies demonstrate that treated cells become sick, and eventually die, over an extended period of time, suggesting an apoptotic mechanism.

OPL98110 Activity On Non Target Cells

OPL98110 was tested on non-target, primary human fetal neurons and a human U251 glioma (astrocytic tumor) cell line. Neurons were activated with TNF-α to simulate inflammation. The glioma cells were aggressively proliferating, and hence, activated. Following a 24 hour exposure to OPL98110 (1:50 dilution) there was no detectable effect on either cell type. Immunohistochemical staining of the neurons for β-tubulin and the detection of apoptosis (TUNEL) revealed healthy, intact cells.

OPL98110 Activity on Migrating Target Cells

To establish whether OPL98110 could distinguish between activated and quiescent cells carefully designed migration experiments were set up using THP-1 cells. In brief, the in vitro migration of activated leukocytes can be induced by chemokines and measured by counting cells that migrate through a 3 μm filter separating the top and bottom chambers of a modified Boyden tissue culture dish. Migration is usually complete in 2 to 3 hours but not every chemokine is an effective chemoattractant even if the cell has the appropriate receptor. For example MCP-3 is a THP-1 chemoattractant but MCP-1 (and hence, OPL98110) is not. Furthermore, a certain percentage of THP-1 cells are constitutively active and will migrate without any specific exogenous stimulus, to a region of low cell density. With suitably long incubation periods, however, it is possible to measure the effects of OPL98110 on the active cells that migrate to the bottom of a Boyden dish.

In our experiments THP-1 cells were plated into the top chamber of modified Boyden dishes and the lower chambers contained culture medium with and without OPL98110. The cells on both sides of the filter were exposed to the chemokine-toxin. After 24 hours the cells in the top and bottom chambers were counted using a Coulter counter. There was no difference in cell numbers in the top chambers between control and tests indicating that equal numbers of cells had migrated under all conditions. In comparison to control, cell numbers in the bottom chambers of treated cells decreased as the concentration of OPL98110 increased. The chemokine-toxin conjugate induced a dose dependent (between 0.5 and 5 μg/ml) decrease in the number of cells in the bottom chamber.

Only the activated (migrating) cells were affected by the chemokine-toxin. For example after 24 hours, approximately 75-80% of stationary THP-1 cells treated with OPL98110 (1:50 dilution) appeared healthy when viewed under the microscope. The mean cell survival rate in migration assays using the same dilution of the chemokine-toxin was 50 $\pm$ 15% (mean of 3 experiments in triplicate).

The over-expression of MCP-1 and target receptors have been observed in a wide range of cancers. For, example the chemokine is responsible for the large leukocyte infiltrates seen in breast, lung and ovarian cancers. MCP-1 has been shown to play a direct role in tumor associated angiogenesis (a first for an β-chemokine family member) and tumor progression. Consistent with this OPL98110 was found to be highly toxic to MCF-7 breast carcinoma cells in culture.

Therapeutic implications

Graduated transient leukocyte modulation therapy (TLMT) Based On The Appearance Of Cell Types

The *in vitro* results demonstrate that the methods and conjugates in this application provide a means to exploit the dynamic nature of chemokine receptor distribution and upregulation that are the hallmark of pathophysiological inflammatory conditions. As described in the application, for a particular

condition, therapy can be initiated with a chemokine receptor-targeting conjugate with the greatest selectivity for the principal leukocyte group involved. For disorders in which there is a time dependent appearance of different leukocyte groups, whose appearance can be monitored by diagnostic tests, additional or different conjugates can be employed in the therapy (TLMT). In each instance of trauma or disease, therapy will be initiated with the chemokine receptor targeting conjugate that has the greatest selectivity for the principal leukocyte subtype involved in that particular form of inflammation. In the case of allergic inflammation of the lung, for example, an Eotaxin-toxin (e.g. OPL98112) would be best suited to target the CCR3 receptors on eosinophils (the principal leukocyte) and any infiltrating Th-2's that may have already appeared. In traumatic injury to the nervous system (both SCI and TBI) a time-dependent appearance of additional cell types will be handled somewhat differently. In the very early stages of SCI and TBI microglia mediate inflammation and they can be targeted with a single chemokine-toxin like OPL98110 (MCP-1 working via CCR2 receptors). If diagnostic testing (analysis of cerebrospinal fluid at the time of first drug administration) indicates that inflammation has progressed to the point where peripheral MNPs and neutrophils are already infiltrating the site of injury, it would be appropriate to supplement OPL98110 treatment with a neutrophil selective agent like IL-8-toxin (OPL00202). If the patient presents with still later stages of inflammation (e.g. several days post injury, which is not unheard of in cases of TBI) then diagnostic testing would likely indicate that peripheral T-lymphocytes have already reached the trauma site and a broader acting chemokine-toxin like OPL98101 (MCP-3) will be required to target the microglia and other MNPs (via CCR1, 2, 3 and 9, but mainly CCR2), neutrophils (via CCR1, 2 and 3, but mainly CCR1), and T-cells (via CCR1,2,3, and 9, but mainly CCR3). Once the inflammation is under better control treatment would continue with OPL98110 (MCP-1, via CCR-2) until all relevant populations of leukocytes have been "modulated" to their pre-injury levels.

For treatment of disorders associated with the nervous system with targeted therapies, since neurons cannot be replaced, the drug cannot target neurons. Hence, a conjugate, such as OPL98110, which targets CCR2 receptors, is an appropriate chemokine-toxin conjugate for use in the nervous system. Patients with traumatic conditions like SCI are likely to present soon after injury; whereas those with chronic inflammation, such as patients with multiple sclerosis and arthritis, may arrive for treatment at various stages of disease. When a typical MS patient transitions from the acute relapsing remitting phase of the disease to the chronic progressive phase, the microglia are activated and transformed into myelin degrading macrophages (Rudick *et al.* (1999) *J Neuroimmunol* **98**, 22-8).

OPL98110 will be a drug of choice for MS in the acute phase and would likely be effective alone. In the chronic phase of MS, monocytes and Th-1 cells constitute most of the infiltrating leukocytes. OPL98110 would be effective against the monocytes but the CXCR3 and CCR5 expressing Th-1 cells will necessitate the addition of a conjugate, such as OPL00203 (IP10) and/or OPL00204 (RANTES), to the treatment regimen.

For arthritis it is not only the stage of disease that must be considered, but also the many subtle differences that this condition can presents. Macrophages and other cell types found in the joint (including endothelial cells) appear to upregulate their production of IL-8 (a powerful neutrophil chemoattractant) in the active phase of rheumatoid arthritis (Takahashi *et al.* (1999) *Tohoku J Exp Med* **188**, 75-87). This indicates that chemokine-toxin conjugates that target the IL-8 producing cells at the very early stages, and those that target neutrophils at a later stage, will be of particular use. The production of IL-8, however, is low in fibrotic synovitis associated with some cases of rheumatoid arthritis, suggesting that the infiltrates do not always contain large numbers of neutrophils. In another twist, Th-1 cells are selectively recruited to the joints of children with juvenile idiopathic arthritis. These cells have upregulated CXCR3 and CCR5 receptors

making them good targets for OPL00203 (IP10 conjugated to shiga toxin) and OPL00204 (RANTES conjugated to shiga toxin).

Broad-Based therapy

The α-chemokine SDF-1β only binds to CXCR4 receptors, but this receptor subtype is found on a very wide array of cell type. In some forms of inflammation, however, a broader approach afforded by a conjugate, such as OPL98111 (SDF1-β- shiga toxin conjugate), is acceptable. It is acceptable, for example, for targeting activated cells in cancer. The α-chemokine SDF-1β only binds to CXCR4 receptors; this receptor subtype is found on a very wide array of cell types, in addition to leukocytes. As noted above, the chemokine-toxin conjugatg OPL98111 targets U251 (astrocytoma), HT-29 (human colon carcinoma), and THP-1 (monocytoid leukemia) cells in culture as well as primary human monocytes T-cells, and primary human neurons. We have also demonstrated that human foreskin fibroblasts and primary fetal astrocytes do not respond to OPL98111.

Xenograft model

Unlike OPL98110, which kills cultured target cells in 12-24 hours, OPL98111 has lytic properties, at least at high concentrations (20 μg/ml lyses a typical target culture in less than five minutes). With this effect in mind a xenograft model used well-established HT-29 tumors in the event that in-vivo potency directly reflected in-vitro potency. The tumors were seeded by injecting one million live HT-29 cells into the right flank of female SCID/CB17 Fox Chase mice and allowed to grow for up to 15 days prior to treatment. This protocol produced heterogeneously sized tumors that made it difficult to measure the changes between control and treated animals. Furthermore, histological examination of similarly sized tumors from treated and control animals revealed that treated tumors contained more dead and dying cells and less evidence of a blood supply.

The initial experimental protocol was far from ideal and all the implanted SCID mice developed very aggressive flank tumors. Despite these shortcomings

in the protocol, OPL98111 retarded tumor growth relative to control animals ($n = 4$ animals per group, $p < 0.0001$, by one and two-tailed Student's t-tests and 2 factor analysis of variance, ANOVA). In a second experiment, the retarding effect of OPL98111 on smaller tumors given earlier treatment was more readily apparent ($n = 3$ animals per group, $p < 0.0001$, Student's t-test) although the great variability of tumor size in control and treated animals continued to obscured the effects of the drug).

Also, toxicology testing indicated that the SDF-1β conjugate, despite the wide distribution of CXCR4 receptors, is less systemically toxic than initially thought. In preliminary toxicology testing, a subcutaneous dose of 5 mg/kg OPL98111 had no apparent effect on normal mice; whereas the same dose given intravenously was lethal within 12-24 hours. A therapeutic dose, given systemically, of a typical ligand-toxin, would generally not exceed 0.25 mg/kg. In the thirty plus days that each xenograft experiment took to complete no animals died and post-mortem histology did not reveal any detectable abnormalities in major organs. Furthermore, histological examination of similarly sized tumors from treated and control animals revealed that treated tumors contained more dead and dying cells (63 v 32 %)and less evidence of a blood supply (no evident vascularization.

The results demonstrated that concerns about broad-acting chemokine-toxins in general, and OPL98111 in particular, appeared to be exaggerated given the tight association between chemokine receptor activation and upregulation in inflammation, and the receptor down regulation that occurs on exposure to the ligand. It appears that these conjugates will be effective at relatively low concentrations and have a somewhat self-limiting effect.

Conclusions

The conjugates may be flexible and powerful therapeutic agents and will have profound effects in cancer applications, among others. They not only can directly target cancer cells but also the activated endothelial cells and leukocyte infiltrates that fuel the disease. OPL98111 and OPL00204 (a RANTES-toxin

conjugate), for example, could have profound effects on HIV infection as they target the CXCR4 and CCR5, respectively. These are the principal viral co-receptors in HIV infection. OPL98112 (eotaxin-toxin conjugate) appears ideal for asthma as its receptor is expressed on the eosinophils and Th2 cells orchestrating the disease. OPL00203 (an IP10-toxin conjugate) would be a good choice in the treatment of MS at the stage where CXCR3 expressing T-cells are the principal leukocytes present.

These agents also have the potential of taking treatment of disease to another level in that the chemokine-toxin selected will be defined by the leukocyte population(s) at a given stage in the pathology of the condition. For example, in the very early stages of traumatic injury to the CNS, microglia mediate inflammation and they can be targeted with OPL98110. If diagnostic testing indicates that inflammation has progressed to the point where infiltrating macrophages and neutrophils are present at the site of injury, it would be appropriate to supplement treatment with a neutrophil selective agent like OPL00202 (an IL-8-toxin conjugate). If the patient presents with still later stages of inflammation where T-lymphocytes will be a component then and a broader acting chemokine-toxin like OPL98101 or OPL00203 would be appropriate. In arthritis, it is not just a question of the stage of disease but also subtle differences in the pathology the this disease can manifest. Macrophages appear to upregulate their production of IL-8 in the active phase of RA. This indicates that chemokine-toxin conjugates that target macrophages at the early stages, and those that target neutrophils at a later stage, would be of particular use. The production of IL-8 is low in fibrotic synovitis associated with some cases of RA, suggesting that the infiltrates do not always contain large numbers of neutrophils. In another twist, Th-1 cells are selectively recruited to the joints of children with juvenile idiopathic arthritis. These cells have upregulated CXCR3 and CCR5 receptors making them good targets for OPL00203 and OPL00204.

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DECLARATION UNDER RULE 132

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

JOHN R. McDONALD

Date: 09/04/00
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